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A HISTOCHEMICAL STUDY OF CEMENT SECRETION DURING THE INTERMOLT CYCLE IN BARNACLES

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Synthesis and accumulation of cement secretion in adult barnacles have been identified in specific regions in cement gland cells of several species (Lacombe, 1968; 1970; Lacombe and Liguori, 1969; Walker, 1970; 1974; Cheung and Nigrelli, 1972). Whether the cement secretion of the gland cells is a continuous or discontinuous process correlated with the physiological condition of the animal, however, has not been reported. Recently criteria for the determination of the molt stages of adult barnacles were developed (Davis, Fyhn and Fyhn, 1973). The present paper is a histological and histochemical study of cement gland cells in two acorn barnacles during the intermolt cycle.

Materials and Methods

Specimens of *Balanus amphitrite* Darwin and *B. eburneus* Gould were collected at the dock of Duke University Marine Laboratory, Beaufort, North Carolina. The animals measured 10 to 15 mm (*B. amphitrite*) and 15 to 25 mm (*B. eburneus*) in basal diameter. Animals were fixed immediately upon collection or after a period of 5 to 31 days in aquaria. Animals were kept at $23 \pm 1^{\circ}$ C in the aquaria at a seawater salinity of 30 ‰. The animals were fed newly hatched *Artemia* nauplii (Metaframe, San Francisco Bay Brand) every second day. The water was changed weekly and at this time shells of the barnacles were cleaned by light brushing. Prior to fixation the animals were molt staged according to the method of Davis, Fyhn and Fyhn (1973). The number of specimens, their molt stage and days kept in aquaria are shown in Table I. The body and opercular valves were removed from the shell after cutting the opercular membrane. The shell together with the mantle tissue, cement glands and ovarioles was fixed for two hours in Carnoy's fixative without chloroform. In addition, Bouin's fluid (48 hours), Rossman's fluid (24 hours) and Carnoy's fixative (2 hours) were used as fixatives. After washing in 100% ethanol (Carnoy, Rossman) or in 70% ethanol (Bouin) the mantle tissues were dissected out, embedded in Paraplast (Fisher Scientific), and serial sectioned at 5 or 8 μ. For general orientation the Mallory-Heidenhain Azan stain (Koneff, 1938) was used. For histochemical studies the methods listed in Table II were applied. Linear dimensions were measured by a *camera lucida* (magnification 813 ×).

Results

The cement gland cells of *B. amphitrite* are located in the mantle tissue among the ovarioles. The cells are rounded or oval with a diameter up to 200 μ. Each gland cell is connected to the cement duct system by a canal (Figure 1). This canal

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TABLE I

Number of specimens studied on Balanus amphitrite *and* B. eburneus *in various stages of the intermolt cycle.*

	Molt stages								Days in aquaria	Month of collection
	A	B_1	B_2	C	D_0	D_1	D_2	Not determined		
B. amphitrite	1	10		11		7	6		5–16	August
B. amphitrite								9	17–31	April
B. amphitrite		1	2		1		2	3	11–26	July
B. amphitrite		1	2	2		2	2		0	October
B. eburneus			1	2	2	1			0–2	August

corresponds to the secondary canal of the cement gland cells in *B. tintinnabulum* (Lacombe, 1968). From the joining point a collector canal runs peripherally in the cement gland cell in an infolding of its plasma membrane. The collector canal is branched or unbranched depending upon the size of the gland cell. The course of the collector canals was determined in serial sections of 25 cement gland cells. The cell size was given by the average of the largest and

TABLE II

Histochemical tests applied to the cement gland cells.

Componnd tested for	Method	Control	Reference
General orientation	Mallory-Heidenhain Azan		Koneff (1938)
Proteins			
General proteins	Mercury-bromphenol blue		Pearse (1968)
Tyrosine containing proteins	Million reaction		Pearse (1968)
Protein bound SS-groups	Thioglycollate-ferric ferricyanide for SS		Adams (1956)
Protein bound SH-groups	DDD for SH	Alkylation	Barnett and Seligman (1952)
Protein bound SS- and SH-groups	Thioglycollate/DDD		Barnett and Seligman (1952)
Carbohydrates			
Acid mucopolysaccharides/Mucins	Toluidine blue 0	RNAse	Pearse (1968), Lillie (1929)
	Azur A	RNAse	Kramer and Windrum (1955)
	Mucihematein	RNAse	Laskey (1950)
	Alcian blue	RNAse	Steedman (1950)
1.2 glycol groups	Periodic acid Schiff's	Acetylation	Casselman (1962), Lillie (1965)
Nucleic acids			
DNA	Feulgen reaction/ Fast green		Feulgen and Rossenbeck (1924)
DNA and RNA	Methyl-green-pyronin Y	RNAse	Kurnick (1955)

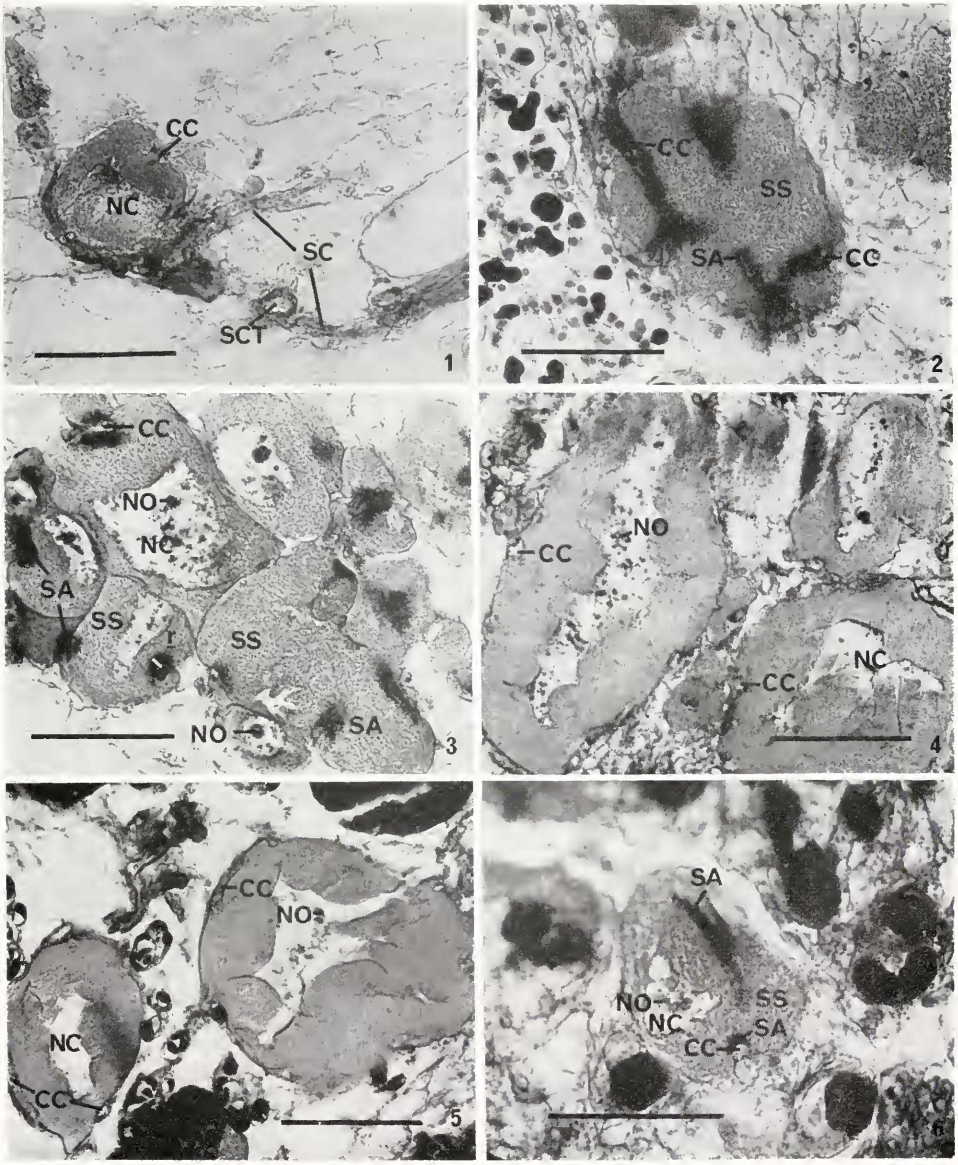


FIGURE 1. Cement gland of *Balanus amphitrite*, stage A, fixed seven days after collection (Azan stains, section 8 μ). CC represents collector canal; NC, nucleus; SC, secondary canal; SCT, secondary canal transversely sectioned; marker is 50 μ.

FIGURES 2 to 6. Cement gland cells of *Balanus amphitrite* in various stages of the intermolt cycle, animals fixed immediately after collection (Azan stain, sections 8 μ). Figure 2 shows stage B_1; Figure 3 shows stage B_2; Figure 4 shows stage C; Figure 5 shows stage D_1; Figure 6 shows stage D_2. CC represents collector canal; NC, nucleus; NO, nucleolus; *r*, width of accumulation area; SA, secretion accumulation area; SS, secretion synthesis area; marker is 50 μ.

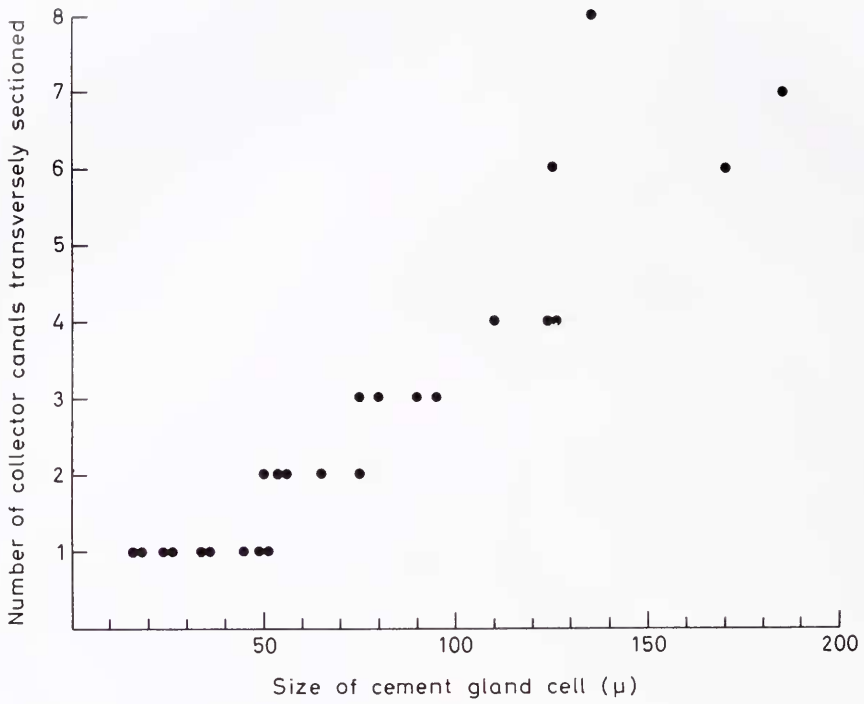
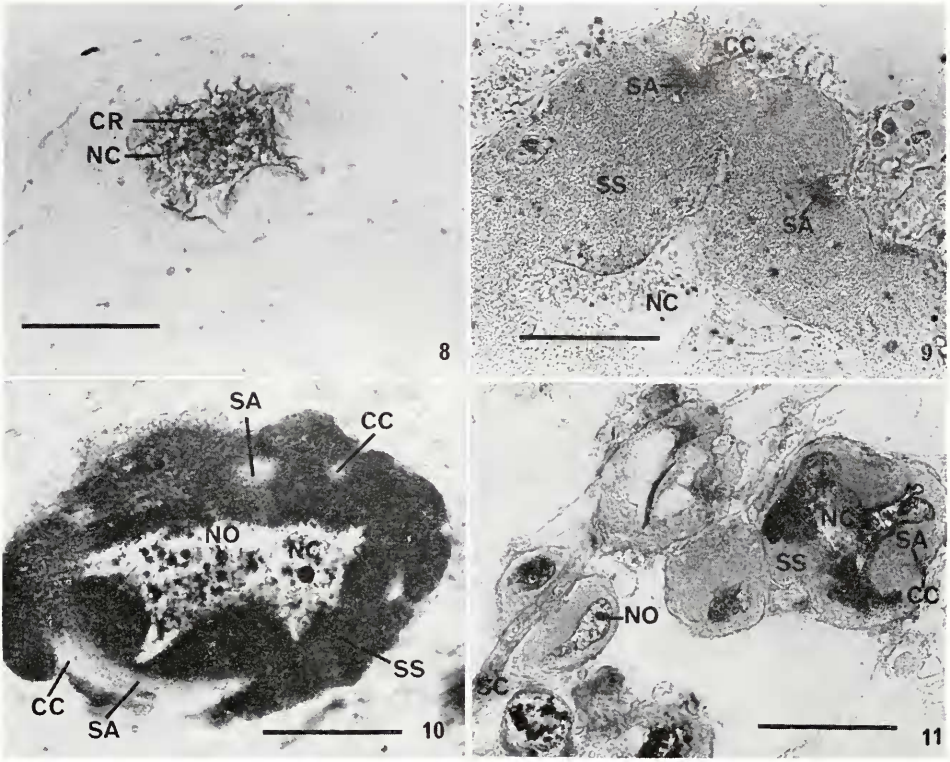


FIGURE 7. Number of collector canals transversely sectioned in cement gland cells of *Balanus amphitrite*. The size of the cells is given by the average of the largest and smallest diameter measured. Each point represents one gland cell.

smallest diameter. In gland cells up to 50 μ the collector canal is unbranched so that only one transversely sectioned canal may be observed. In cells of 50 to 75 μ the collector canal encircles the cell allowing the canal to be sectioned twice (Figure 3). In cells of 75 to 100 μ the collector canal appears to branch once so that three canals in transverse section were observed. In cells of 100 to 125 μ four transversely sectioned canals were observed. In cells of more than 125 μ the branching is more complex and the path of the collector canals is undulated which complicates the accurate determination of the number of canals. A minimum of six transversely sectioned canals were observed in serial sections of these cells. None of the collector canals seemed to have a "dead-end". The number of transversely sectioned collector canals in cement gland cells of various sizes are summarized in Figure 7.

The nucleus of cement gland cells up to 50 μ is rounded with a diameter of about 50% of the cell diameter (Figure 3). In larger cells the nucleus is irregular in outline making up 50 to 90% of the cell diameter in cross sections (Figures 3 and 10). The nuclei are rich in chromatin (Figure 8). In gland cells of less than 50 μ, one nucleolus was observed (Figure 3); in larger cells there are numerous nucleoli of various sizes (Figure 10). The cytoplasm appears dense and finely granulated without vacuoles. Sporadically, vacuoles were observed in the cytoplasm of gland cells fixed in Bouin's fluid. This is assumed to be a fixation artifact since Bouin's



FIGURES 8 to 10. Cement gland cells of *Balanus amphitrite* fixed immediately after collection (sections 8 μ). Figure 8 shows a cell in stage C stained by the Feulgen reaction; Figure 9 shows a cell in stage B_1 stained by Mercury-bromphenol blue; Figure 10 shows a cell in stage B_1 stained by Toluidine blue O. CC represents collector canal; CR, chromatin; NC, nucleus; NO, nucleolus; SA, secretion accumulation area; SS, secretion synthesis area; marker is 50 μ.

FIGURE 11. Cement gland cells of *Balanus eburneus*, stage B_2, fixed two days after collection (Azan stain, section 8 μ). CC represents collector canal; NC, nucleus; NO, nucleolus; SC, secondary canal; SA, secretion accumulation area; SS, secretion synthesis area; marker is 50 μ.

fluid has been reported to give vacuoles in a variety of tissues (Humason, 1972).

In animals fixed immediately after collection (Table I) the staining properties of the cytoplasm of the cement gland cells varied with the molt stage of the animal (Figures 2 to 6). In the postecdysial stage B_1 (Figure 2), two distinct regions within the cytoplasm were discerned by Azan staining: the region adjacent to the collector canals stained bright red while the remainder of the cytoplasm stained yellow to orange. These two regions correspond to the region of secretion accumulation and region of secretion synthesis, respectively, described for other barnacles (Lacombe, 1970; Walker, 1970). The size of the red areas was determined in cement gland cells of specimens of *B. amphtrite* in various stages of the intermolt cycle. The size was expressed by the width (marked by an *r* in Figure 3) of the red areas of transversely sectioned collector canals. The red areas do not change in width along the canal (Figure 2). No correlation was found between the

TABLE III

Width (μ) of secretion accumulation areas in cement gland cells of Balanus amphitrite *in different stages of the intermolt cycle. (The data are given as mean ± SE with the range in parenthesis of the 10 largest widths measured in 10 gland cells from each animal. Animals were fixed immediately after collection with Azan stain.)*

Animal number	Molt stage				
	B_1	B_2	C	D_1	D_2
1	11.3 ± 0.60 (9–15)	7.9 ± 0.28 (7–9)	0	0	3.7 ± 0.34 (2–5)
2	— (0–4)	1.2 ± 0.55	0	0.9 ± 0.02 (0.7–0.9)	2.3* ± 0.33 (2–3)

* Mean of three measurements.

width and the cell size. Table III shows the mean width of the ten largest areas measured in each animal. The size of the red areas was largest in stage B_1, decreasing during stage B_2, was zero in stage C and again increasing from D_1 to D_2. The areas staining red with Azan showed positive reactions in the protein tests (Table IV and Figure 9), but showed negative reactions for the carbohydrates tested (Table IV). The remainder of the cytoplasm showed negative reactions in the tests for proteins (Figure 9) and carbohydrates, and showed a strong positive reaction for RNA (Methyl-green-pyronin Y). This area stained dark blue by Toluidine blue O (Figure 10) and Azur A, and blue-green by Alcian blue (Table IV). After treatment with ribonuclease the cytoplasm was negative for Toluidine blue, Azur A and Alcian blue, indicating that the reactions in these tests were due

TABLE IV

Histochemical reactions of the cytoplasm of cement gland cells in Balanus amphitrite *showing secretion accumulation.*

Method	Secretion accumulation area	Secretion synthesis area
Azan	Red	Yellow-orange
Mercury-bromphenol blue	xxx	–
Millon reaction	x	–
Thioglycollate ferric ferricyanide	xx	–
DDD/Control	x/–	–/–
Thioglycollatic reduction, DDD	xx	–/–
Toluidine blue 0/Control	–/–	Dark blue/–
Azur A/Control	–/–	Dark blue/–
Mucihematein/Control	–/–	x/–
Alcian blue/Control	–/–	Blue-green/–
Periodic acid Schiff's/Control	–/–	–/–
Feulgen reaction	–	–
Methyl-green-pyronin Y/Control	–/–	xxx/–

xxx Strongly positive reaction
xx Moderately positive reaction
x Weakly positive reaction
– Negative reaction

to RNA. No true metachromasia was observed. In gland cells not showing red areas the cytoplasm adjacent to the collector canals was negative for proteins, RNA and carbohydrates. The remainder of the cytoplasm showed histochemical reactions similar to those found for the synthesis region in cells showing red areas (Table IV).

In *B. amphitrite* maintained in aquaria before fixation (Table I, Figure 1), no areas stained red by Azan were observed in cement gland cells irrespective of the size of the cement gland cell, the molt stage of the animal, or the number of days in aquaria (5 to 31 days). The gland cells showed the same histochemical reactions as cells lacking red areas in animals fixed immediately after collection. No difference was observed between gland cells of animals fixed in the different fixatives or in animals collected during spring and summer.

In *B. eburneus* the cement gland cells and the duct system had a histology similar to that found in *B. amphitrite* (Figure 11). The collector canals were running extracellularly in infoldings of the plasma membrane and the number of branches of collector canals increased with the cell size. Up to nine transversely sectioned canals were observed in the largest cells (diameter up to 180 μ). Small areas staining red by Azan were observed in cement gland cells of animals in stage B_2 (Figure 11) fixed two days after collection (width 2.9 ± 0.28 μ) and D_1 fixed after one day (width 4.4 ± 0.16 μ). No red areas were found in animals of stage C fixed after one day, and D_0 fixed immediately and after two days. The two regions of the cytoplasm showed histochemical reactions similar to those found for *B. amphitrite*.

Discussion

The cytology of the cement gland cells in adult specimens of *B. amphitrite* and *B. eburneus* found in the present study is mostly in agreement with earlier descriptions of these species (Lacombe, 1970; Cheung and Nigrelli, 1972). The collector canals, however, were shown to be running extracellularly in infoldings of the plasma membrane and showed an increase in ramification with increasing size of the gland cell (Figure 7). Secretion accumulation could be found to an equally large degree along the collector canals in both species (Figure 2). More detailed investigations are necessary to determine whether the collector canals break up further into intracellular ducts as found in *B. balanoides* and *Elminius modestus* (Walker, 1970). The two regions in gland cells of *B. amphitrite* and *B. eburneus* showed histochemical reactions similar to those of the regions of secretion synthesis and secretion accumulation described for *B. eburneus* (Cheung and Nigrelli, 1972) and for *B. balanoides* and *E. modestus* (Walker, 1970).

Secretion accumulation in *B. amphitrite* seems to be correlated with the intermolt cycle. The accumulation could result from a synthetic activity starting in early proecdysis (D_1), increasing in D_2, reaching its maximum around ecdysis or in the postecdysial stage B_1, and decreasing to zero during B_2. The cyclic changes in the secretion accumulation could then reflect similar changes in the flow rate of secretion from the cement gland cells. In interecdysis (stage C) synthesis or accumulation seems absent. The amount of RNA in the synthetic region of cement gland cells was high in all molt stages, and no increase in protein concentration in this region could be detected in stage B_1. The applied techniques, however, do not adequately distinguish between small concentration differences. It seems unlikely that the

accumulation should result from variations in flow rate of secretion from the cell with the secretion synthesis being constant, since not even traces of secretory product were observed adjacent to the collector canal in stage C animals (Figure 4).

In *B. eburneus* secretion accumulation was observed in animals in stage B_2 and D_1, but not in stage C and D_0. The areas were smaller than those appearing from earlier reports (Lacombe, 1970; Cheung and Nigrelli, 1972), but showed the same histochemical reactions as described for this species (Cheung and Nigrelli, 1972). The areas in *B. eburneus* in stage B_2 and D_1 were comparable in size to the areas in *B. amphitrite* in the same stages, and a molt cycle dependency of secretion accumulation is therefore possible also in this species.

The structural changes in the integument of adult *B. amphitrite* during the intermolt cycle (Davis, Fyhn and Fyhn, 1973) are similar to those described for malacostracans (Passano, 1960; Yamaoka and Scheer, 1970). The increasing amount of secretion in cement gland cells is thus coinciding with the deposition of the new exoskeleton. This raises interesting questions concerning the control of cement secretion in barnacles.

In specimens of *B. amphitrite* maintained in aquaria for 5 to 31 days before fixation, no secretion accumulation was observed in cement gland cells regardless of the molt stage of the animal. Animals in crowded populations of *B. balanoides* being lifted up from the substratum show cement droplets at their bases (Darwin, 1854). In *B. crenatus* and *B. glandula* cement was extended onto the base of animals separated from the substratum (Saroyan, Lindner and Dooley, 1970). Walker (1972) obtained droplets of cement from the base of *B. crenatus* and *B. hameri* kept with an airfilled space between the base and an underlying glass plate. In the present study no extended cement was observed on the bases of animals kept in aquaria. Newman, Zullo and Wainwright(1967), referring to unpublished data, reported *B. amphitrite* to reattach to glass slides. It is obscure why captivity apparently inhibited the secretory activity of the cement gland cells of *B. amphitrite* in the present study. However, it is possible that a minimum of contact between the animal and the substratum is required for cement secretion and extension to occur. A seasonal dependence of cement secretion can not be excluded since animals showing accumulation were collected in October, while animals not showing accumulation were collected during spring and summer. The present study shows that detectable secretion accumulation of cement gland cells in adult barnacles is not necessarily a species characteristic, but may be dependent upon exogenous as well as endogenous factors.

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Summary

1. Regions of secretion accumulation and secretion synthesis were found in the cytoplasm of cement gland cells of adult *Balanus amphitrite* and *B. eburneus*. In contact with the accumulation regions collector canals were running extracellularly in infoldings of the plasma membrane and showed increasing ramification with increasing size of the cement gland cell.

2. In *B. amphitrite* the secretion accumulation was at its maximum in stage B_1 of the intermolt cycle, decreased during stage B_2, was zero in C and increased from D_1 to D_2. A similar variation in *B. eburneus* seemed probable.

3. The histochemistry of the accumulation and synthesis areas was studied.

4. In *B. amphitrite* maintained in aquaria before fixation, no secretion accumulation was observed in cement gland cells irrespective of molt stage of the animal.

5. The study shows that detectable secretion accumulation in cement gland cells of adult barnacles is not necessarily a species characteristic, but may be dependent upon endogenous as well as exogenous factors.

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